Cell, Vol. 111, 143–146, October 18, 2002, Copyright ©2002 by Cell Press

Endocytosis: Driving Membranes around the Bend

Minireview

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When a nascent vesicle buds, the membrane must curve. Several mechanisms have been proposed for curvature creation or stabilization. Structural analysis of the ENTH domain of the endocytic protein epsin has suggested a new mechanism, in which the ENTH domain pushes its way into membranes, thus bending them into shape.

The plasma membrane of cells presents a barrier to the entry of large water-soluble particles and nutrients, such as iron, folate, and LDL-cholesterol. This barrier is overcome through endocytosis, the process by which portions of the plasma membrane and extracellular fluid are taken up into a cell. Endocytosis is also critical for many physiological responses; for instance, downregulation of certain signaling receptors. Endocytosis can occur via several distinct pathways. The best-characterized pathway depends upon the cytosolic protein complex clathrin, along with its accessory factors (reviewed in Brodsky et al., 2001; Kirchhausen, 2000). The process begins by gathering together plasma membrane proteins and lipids through interactions with cytosolic adaptors and accessory factors. Some of these cytosolic factors bind to clathrin and stimulate its polymerization into a spherical basket of pentagons and hexagons. As the endocytic vesicle forms, the plasma membrane and associated proteins and adjacent extracellular fluid are pulled inward into the cytosol. Upon scission, the clathrin-coated vesicle is released into the cytoplasm (Figure 1).

Clathrin accessory factors generally exhibit multiple protein interaction domains that allow them to form a web of contacts. Clathrin-coated vesicles are not highly enriched for accessory factors, thus, these factors are not structural, but rather perform regulatory or catalytic functions at specific stages in endocytosis. One accessory factor, epsin, was discovered in 1998 by virtue of its binding to another accessory factor, eps15 (Chen et al., 1998). Epsin has become a focus of interest due to its interactions with membranes, accessory proteins, clathrin, ubiquitin, and possibly certain cargo proteins (Wendland, 2002).

Membranes Get Bent out of Shape

How do changes in curvature of the membrane bilayer occur during bud formation and resolution of the coated pit into a vesicle? The process of clathrin polymerizing into a basket, linked to the membrane via adaptor proteins, is thought to physically pull the membrane inward toward the cytosol to initiate formation of an endocytic vesicle. Later, the accessory proteins amphiphysin, endophilin, and dynamin are concentrated at the narrow, highly curved membrane "necks" of deeply invaginated clathrin-coated pits, where they function in the final scission step that converts the coated pit into a coated vesicle. The De Camilli and Hinshaw labs found that these three proteins can each, on their own, promote the tubulation or vesiculation of membranes (reviewed in Hinshaw, 2000). This is consistent with amphiphysin, endophilin, and dynamin directly altering the conformation of the lipid bilayer. Various mechanisms for this process have been proposed, ranging from oligomerization-driven mechanical deformation of the lipid bilayer by dynamin to enzymatic lipid remodeling activities of endophilin that alter the biophysical properties of the lipid bilayer.

In order for a protein to effect membrane curvature, it must first bind to membranes. Each of these tubulating proteins binds to membranes, often by interacting specifically with the phosphoinositide PtdIns(4,5)P_2 (PIP_2). Recombinant dynamin, amphiphysin, or endophilin can transform artificial liposomes containing acidic phospholipids and/or PIP_2 into protein-wrapped tubules. It has been suggested that dynamin promotes scission through a combination of binding activities, GTP-driven conformational changes of the dynamin oligomer, and additional functions of associated proteins that conspire together to promote fission of the membrane (Hinshaw, 2000).

One concern about the biophysical contortions of the membrane at the constricted neck is the high degree of curvature, both positive (around the circumference of the neck) and negative (the sharp bend as viewed perpendicular to the plasma membrane) (see Figure 1). One model that tried to account for these extremes in curvature suggested that the enzymatic activity of endophilin (the condensation of lysophosphatidic acid + acyl CoA to form phosphatidic acid) altered the curvature of the membrane by physically changing the composition of the phospholipids from "inverted cone shaped" to "cone shaped," and thus affected the inherent disposition of the membrane (Schmidt et al., 1999). However, this model is now disfavored since endophilin lacking the enzymatic activity can still tubulate membranes (Farsad et al., 2001). These final scission events are likely to require quite different changes in membrane structure and curvature as compared to those that initiate bud formation, to which we now turn our attention.

ENTH Domains

Studies of epsin by the De Camilli lab (Chen et al., 1998) and other groups have indicated a role for epsin in endocytosis, in particular the highly conserved amino terminal region (Wendland et al., 1999), which was christened the epsin N-terminal homology (ENTH) domain (Kay et al., 1998). While many studies have indicated a central role for epsin in the process of endocytosis, the precise

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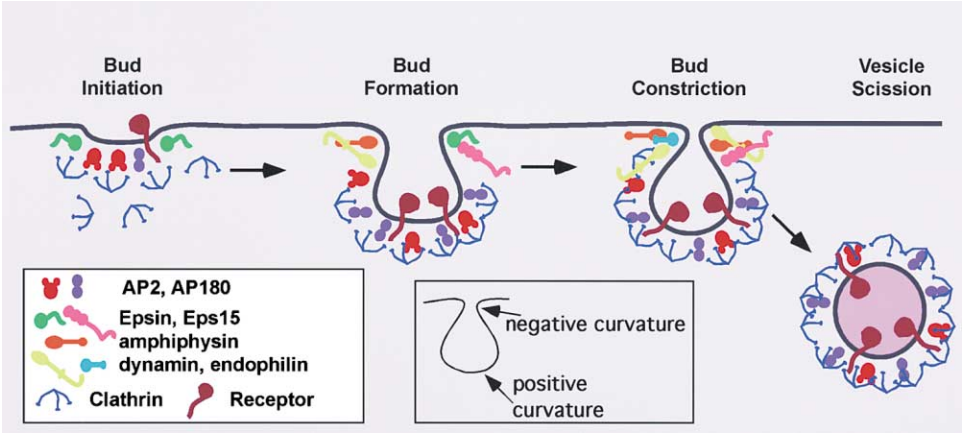

Figure 1. The Process of Endocytosis Is Depicted as a Series of Events Beginning with the Initiation of a Bud and Culminating in the Release of a Clathrin-Coated Vesicle into the Cytoplasm

Bud initiation is thought to occur in part by the association of epsin as well as adaptor proteins like AP2 binding sorting signals on receptor tails. As the bud forms and constricts, the activities of other proteins such as endophilin and amphiphysin cooperate at the edge of the growing clathrin coat to remodel the lipids at the bud neck. The GTPase activity of dynamin is required for the scission event that separates the vesicle from the plasma membrane. Negative and positive curvature are indicated by arrows in the inset.

functions of epsin and its ENTH domain have remained elusive (Wendland, 2002). McMahon and colleagues have re-examined the structure of the epsin ENTH domain, and their findings suggest that at least one function of epsin may be to initiate the budding process (Ford et al., 2002).

The first look at epsin's ENTH domain was obtained two years ago (Hyman et al., 2000). In the absence of ligand, the ENTH domain has a structure that is simple and elegant. It is an almost perfect superhelix of seven helices, with an eighth helix misaligned with the superhelical axis. The ENTH domain is related in structure to another superhelical trafficking motif, the VHS domain. The similarity appears to end there: the primary sequences of ENTH and VHS domains are not homologous, and their known biological functions are different. Last year, three additional reports on ENTH domain structures emerged. The ENTH domains of AP180 (Mao et al., 2001) and the AP180 homolog CALM (Ford et al., 2001) both turned out to be extended, by two helices in the AP180 fragment, and by one in CALM. All of the helices fit the superhelical pattern, so in this respect the AP180 and CALM domains are more reminiscent of VHS domains. McMahon and coworkers were able to solve the CALM structure in complex with a short-chain PIP_2 lipid, as well as with two soluble inositol polyphosphates. The structures revealed contacts between 3 Lys and a His side-chain and the phosphates of the headgroup. In comparison, 9–12 hydrogen bonds are found in high affinity complexes of phosphoinositides with PH domains (reviewed in Hurley and Misra, 2000). Thus, the ~4 hydrogen bonds in the CALM/IP_3 complex are few in number and unusually exposed to solvent, which is completely consistent with the moderate affinity and specificity for PIP_2.

In the third ENTH domain structural study, Itoh et al. (2001) found that the epsin ENTH domain binds PIP_2 with higher affinity than AP180 or CALM. They went on to anaylze NMR chemical shift changes in the epsin ENTH domain when bound to the headgroup of PIP_2, IP_3. They came to the surprising conclusion that the epsin ENTH binding site for IP_3 was different from the site on CALM and AP180. This finding was unexpected, but not unprecedented. The PIP_2 binding sites on the PH domains of phospholipase C-δ and spectrin, for example, are in different places (reviewed in Hurley and Misra, 2000). The most surprising result was the contrast of the high affinity of the interaction versus the small number of hydrogen bonds observed between the ENTH domain and the phosphoinositide headgroup.

Enter the latest chapter in ENTH domain structural biology. McMahon and his colleagues succeeded in crystallizing the epsin ENTH domain bound to IP_3. The epsin ENTH domain, when bound to its ligand, has nine helices rather than the eight seen for the unliganded structure. The additional helix, termed helix 0, is amphipathic and has a remarkable feature: for a piece of a soluble protein it is turned inside-out. The polar residues line the inside face of the helix, where it is glued to the rest of the ENTH domain via the IP_3. Reversing the pattern of water-soluble protein structures (but conforming to that of membrane proteins), it is the hydrophobic residues of helix 0 that face the exterior.

Welcoming ENTH to the Family of Membrane-Penetrating Domains

The ENTH domain/IP_3 complex fits beautifully into a pattern set by other membrane targeting domains. For example, the protein kinase Cs (PKCs) are a family of signaling enzymes that tightly associate with and penetrate membranes upon activation. The structure of the diacylglycerol and phorbol ester binding C1 domain of protein kinase C shows how a large hydrophobic surface on one face of the domain penetrates deeply into membranes when the activator is bound (reviewed in Hurley and Misra, 2000). Likewise, the phosphatidylinositol 3-phosphate (PI(3)P) binding site of the FYVE domain is aligned with a hydrophobic tip such that the hydrophobic surface can penetrate membrane bilayers when

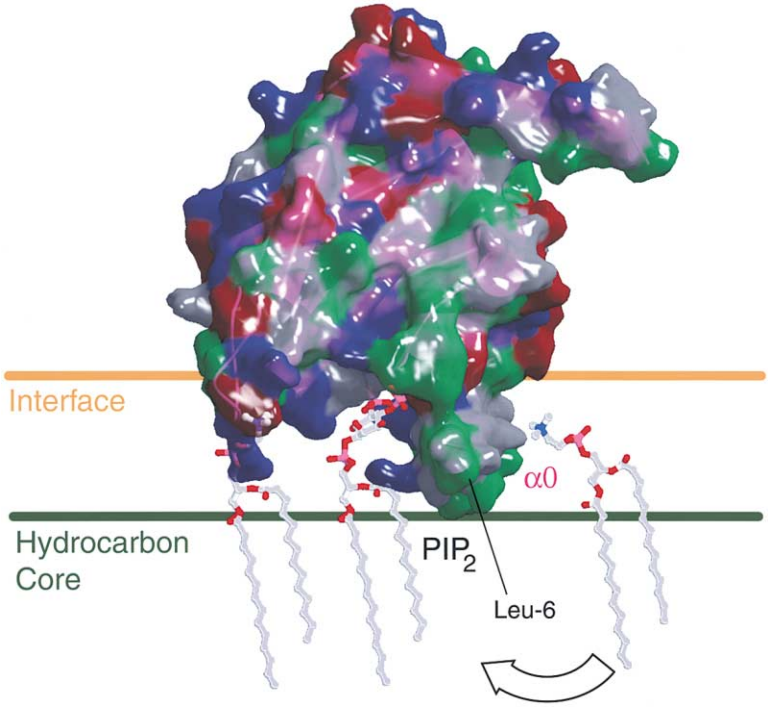

Figure 2. How epsin-ENTH Might Insert into a Bilayer Membrane

The protein is shown with a translucent surface representation, colored green for hydrophobic; blue, basic; red, acidic; and gray, uncharged polar. The membrane is shown in a schematic representation, with the 15 Å thick interface region drawn to scale. Two dimyristoylphosphatidylcholine molecules are shown to indicate scale and potential nonspecific interactions between the ENTH domain and surrounding lipids. The large open arrow near one of the phosphatidylcholine molecules suggests the direction in which the lipid tails would need to repack to accommodate the inserted helix 0 of the ENTH domain, which would potentially promote curvature.

PI(3)P is bound (Hurley and Misra, 2000). In the predicted ENTH/PIP_2 complex, the lipid tail points in the same direction as the exposed hydrophobic face of helix 0, leading to a consistent model for membrane penetration (Figure 2).

The ENTH structure differs from the above-mentioned domains in that it is a single amphipathic helix that penetrates the membrane. This helix is conceptually separable from the rest of the protein. Many proteins contain amphipathic helices that bind to and penetrate bilayers in order to target these proteins to appropriate membranous loci in cells. Certain naturally occurring peptide toxins, such as mellitin, also interact with and penetrate membranes as part of their biological function. Mellitin is perhaps the single best studied model system for the effects of insertion of an amphipathic helix on bilayer structure. At high concentrations, mellitin oligomerizes and forms membrane-permeabilizing pores. At low concentrations, individual mellitin helices insert into the bilayer interface with the helix axis parallel to the surface of the membrane, much as proposed for helix 0 of the epsin ENTH domain. Under these conditions, mellitin monomers produce modest perturbations of membrane structure upon insertion (Hirstova et al., 2001).

What are the biological functions of membrane penetration? Lipid-dependent targeting domains such as C1 and FYVE have hydrophobic surfaces surrounding the binding site for the specific lipid activator. These hydrophobic surfaces interact non-specifically with the hydrocarbon core of the membrane. The interactions gained by membrane penetration greatly enhance the affinity for the membrane, by factors of as much as 10^4. In a counterexample, the PH domain of phospholipase C-δ lacks such hydrophobic surfaces, does not significantly penetrate membranes, and binds soluble IP_3 several-fold better than PIP_2. The extent of a domain's ability to penetrate membranes can therefore contribute to membrane affinity and be a driving force for targeting.

Signaling proteins that have membrane-penetrating domains—PKC is the most famous of these—have long been known to be sensitive to changes in membrane biophysical properties, including modulation of curvature, as shown by Epand, Sando, Stubbs, and others (reviewed in Stubbs and Slater, 1996). Now McMahon and coworkers have turned the equation around. They postulate that in the course of shoving its way into the membrane, the ENTH domain pushes surrounding lipids aside, producing a more curved surface. Instead of merely communicating news of changes in membrane physical properties to downstream signal receptors, the ENTH domain—like the gonzo journalist Hunter S. Thompson—is itself creating the news. Now the ENTH domain is an inducer rather than a mere reporter of curvature stress.

Bend it Like epsin

One of the biggest surprises from Ford et al. (2002) was the ability of epsin to stimulate tubule formation in the absence of oligomerization. The proposed mechanism by which epsin induces membrane bending is quite different from previous models; in this case, when the epsin ENTH domain binds its favored ligand PIP_2, an amphipathic helix is induced to fold. This helix formation in turn causes membrane curvature when it becomes buried in the cytoplasmic leaflet of the bilayer (see below). How well does this new model fit with what we know about membrane mechanics?

With the ENTH domain, the question arises whether effects on targeting efficiency can be separated from effects on membrane penetration. The challenges in dissecting these functions center on the difficulty of modulating one membrane biophysical property without another. One of the most convincing ways to do this is to

find mutants in which the effects are uncorrelated. Many of the mutations, e.g., of basic residues in the PIP_2 binding site, produce the expected effects: loss of in vivo targeting and function, loss of membrane binding, and loss of membrane tubulation activity. On the other hand, the ENTH L6W mutant protein reduces membrane affinity and decreases targeting efficiency, but it actually is better than the wild-type protein at promoting tubulation. These observations support the concept that different physical forces are at work in targeting versus tubulation.

Curvature induction can be conceptualized in terms of microscopic interactions at the atomic level, as above, or in a macroscopic formalism in which the membrane is treated as a bulk material. The latter is the more helpful when it comes to estimating the energetics of the process. The free energy per unit area of membrane curvature is $F = 2B(1/R)^2$ for a symmetric membrane, where B is the elastic curvature modulus and R is the radius of curvature. A back-of-the-envelope calculation suggests a curvature free energy cost of 300 kcal/mol to completely bend a planar bilayer into a sphere (Nossal and Zimmerberg, 2002). While this may seem like a lot for a single molecular event, the free energy change on a per lipid basis is much smaller, given perhaps ~20,000 lipids in a 50 nm diameter vesicle. Thus the change in the physical environment and conformation of individual lipid molecules, on average, need not be great. However, budding is a cooperative phenomenon, in which a simultaneous rearrangement of lipids must be involved, occurring with low probability if the membrane is acted upon only by thermal perturbations (Nossal and Zimmerberg, 2002). In the case of a chemically asymmetric membrane, there can be an imbalance between the interfacial tension and the internal surface pressure of the membrane. As shown in a set of classic 1974 papers by Singer and Sheetz, Evans, and Helfrich (see Nossal and Zimmerberg, 2002, for original references), this asymmetry can drive an expansion or contraction that leads to spontaneous curvature. The insertion of epsin ENTH domains into the cytoplasmic face of the plasma membrane could increase the surface pressure on one side of the membrane, creating an imbalance and lowering the curvature energy. If epsin is only involved in the initial bud formation, the total area of the deformed membrane will be much less than that of the mature vesicle. In this case, a handful to a few dozen epsin molecules might be enough to shift the balance to favor the curved state, even if each insertion event only stabilized curvature by as little as 1 kcal/mol. Consistent with this, studies on membrane deformation by mellitin suggest that the effects of inserting individual helices are small. By the same token, the energetic cost of the deformation is not great. The energy estimates suggest that it is not hard to induce curvature in membranes, and membrane penetration seems as plausible a mechanism as any to accomplish this.

A General Mechanism for Membrane Curvature Induction?

How general could this proposed mechanism for membrane curvature be? Is insertion of an amphipathic helix into a leaflet of a bilayer the only way to skin this cat? The tubulating activity of endophilin requires the amphipathic properties of helical region, similarly to the amphipathic helix of the ENTH domain of epsin. Perhaps the proposed mechanism of action of the ENTH domain in membrane curvature is not so unique after all! The similarities between amphipathic helices in epsins, endophilins, and amphiphysins suggest that this could be a commonly used way to bend a membrane. However, given the inventiveness of nature, it is quite likely that other mechanisms exist to produce architectural changes in a bilayer. Polymerization of actin at the cortex of the cell might be capable of physically bending a membrane. Proteins such as caveolin may form invaginations through a combination of membrane insertion and oligomerization. Modulation of membrane lipid composition may well regulate curvature directly. For example, phospholipase A_2 is required for tubule formation from multiple organelles (de Figueiredo et al., 1998). The enzyme action of endophilin, although dispensable for tubulation, might still play a role at some stage (Schmidt et al., 1999). Other examples of epsin-independent budding events range from COPII-mediated budding at the ER (perhaps mechanically driven as originally proposed for clathrin?) to the exciting new area of "topologically backward" vesicle budding from the cytoplasm into the lumen of a forming multivesicular body. Curvature is complicated, and there will be a few more bends in the road before the story is all told.

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